Inactivation of SARS-CoV-2 in liquid phase: are Aqueous Hydrogen Peroxide and Sodium Percarbonate Efficient Decontamination Agents?

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SUPPLEMENTARY INFORMATION

Experimental Details

Two mother solutions of hydrogen peroxide at 6.64 % ± 0.04 (wt./wt.) and 1.02 % ± 0.02 (wt./wt.) were prepared diluting a 35.1% stabilized H_2O_2 solution (Sigma-Aldrich, product no. 18304-1L; lot no. STBJ3885) with sterile ultrapure deionized water (Merck, MilliQ, 10^{18} MΩ cm^{-1}) and stored at +4°C until use. H_2O_2 content was determined by volumetric iodometric titration: aliquots containing

ca. 30 mg of H_2O_2 were added to a potassium iodide (Sigma-Aldrich; puriss. p.a.) aqueous solution, acidified by sulfuric acid (Carlo Erba; puriss., for analysis), and titrated with 0.1 M sodium thiosulfate pentahydrate solution (Carlo Erba; RPE, for analysis), detecting the end point without starch indicator.

An aqueous solution of sodium percarbonate (SP) was freshly prepared by dissolving $Na_2CO_3 \cdot 1.5H_2O_2$ (Aldrich, product no. 371432-2.5KG; lot no. MKBV7961V; H_2O_2 content in the solid: 27.1 % wt./wt.) in sterile ultrapure deionized water. The obtained mother solution 1.00 ± 0.02 % (wt./wt.) was immediately used after measuring its H_2O_2 content.

The peroxide-containing aqueous solutions to be tested were mixed in a 1:1 ratio with the virus suspension, so that the final concentration of the biocidal agent was 50% of the pristine concentration. In the final solutions named HP-3, HP-0.5 and SP the peroxide contents were 3.32 % wt./wt., 0.51 % wt./wt. and 0.51-0.53 % wt./wt., respectively. Uncertainties for these values were around ± 0.02 % (wt./wt.). A SARS-CoV-2 viral suspension was mixed with sterile distilled water (1:1 ratio) and used as positive control of untreated virus.

Aqueous solutions of 0.187 M sodium carbonate decahydrate (Sigma-Aldrich; puriss. p.a., ≥99.0%), 3.12 M of acetic acid (Sigma-Aldrich; ReagentPlus, ≥99%) and 1.04 M citric acid (Sigma-Aldrich; 99.0%) were prepared with sterile ultrapure deionized water and used to adjust the test media pH to the desired value.

HP-3 and HP-0.5 solutions at pH 7.3 and SP solution at pH 10.5 were used as such, without any addition of further pH-adjusting agents.

HP-3-C at pH 2.5 and HP-3-A at pH 3.6 were obtained by adding citric acid or acetic acid, respectively, to the HP-3 formulation.

The potential presence of peroxoacids in HP-3-C and HP-3-A solutions was checked by differential ceriometric / iodometric titration [37]. Briefly, the content of hydrogen peroxide in the solution aliquots was titrated by ceriometric titration, with 0.1 M $Ce(SO_4)_2$ (Aldrich) in aqueous 5 % vol/vol H_2SO_4, by using ferroin as an indicator, previously prepared by addition of $FeSO_4$ (Carlo Erba, puriss.) to 1,10-phenanthroline monohydrochloride monohydrate (Merck-Sigma, 99%). Conversely, the content of hydrogen peroxide plus (potential) peroxoacids were titrated by iodometric titration, as described above. The difference between the peroxide content revealed by ceriometric titration and the one measured by iodometric approach corresponds to the content of peroxoacids (either peroxocitric or peroxoacetic acid). Each titration result was mediated on three experimental values.

^{13}C Nuclear Magnetic Resonance spectra were recorded at 297 K over a 400 MHz Bruker UXNMR Spectrometer in 1 mL of deuterium oxide (D_2O, 99.9 atom % D, Sigma-Aldrich/Merck) and placed in 5 mm-wide NMR tube.

Contact times of 5, 10 and 15 min were chosen to simulate surfaces sanitizing conditions in common household practice. Each virucidal solution was tested in triplicate. At the end of contact time, an aliquot of lyophilized catalase (Sigma; from bovine liver; lyophilized powder; product no. C9322; lot no. SLBT6857; 4419 $units_{protein}$ mg^{-1}) suspended in 50 mM potassium phosphate buffer at pH 7.0 was added to each test solution in order to decompose any residual H_2O_2 and stop the test. Either sodium carbonate or citric acid was added to adjust pH to neutral level before the addition of catalase. Considering that 1 unit of catalase can decompose *ca*. 1.0 mmol of H_2O_2 at neutral pH and 25°C, aliquots of catalase with a 15- or 10-fold stoichiometric excess of the enzyme to the maximum H_2O_2 content were used. The efficacy of the catalase action was confirmed by iodometric titration and, in all cases, no detectable amounts of residual H_2O_2 were found.

All pH values of the aqueous solutions were measured with a pH-meter (AMEL instruments; mod. 338) equipped with a glass electrode.

SARS-CoV-2 virus stock was prepared propagating the virus isolate (GenBank accession number: MW000351) in Vero E6 cells culture (ATCC® CRL-1586™). In brief, Vero cells were cultured in Dulbecco's Modified Eagle Medium with L-glutamine (DMEM, Gibco™ ThermoFisher Scientific), supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Gibco™ ThermoFisher Scientific) and 1% Penicillin-Streptomycin [5,000 U mL^{-1}] (Pen-Strep, Gibco™ ThermoFisher Scientific) and incubated at 37°C and 5% CO_2 atmospheric pressure in a 75 cm^2 cell culture flask, until 80% to 90% of cell confluence; one mL of virus isolate plus 9 mL of medium (DMEM at 2% FBS and 1% Pen-Strep) were then added, incubating the flask at 37 °C with a CO_2 level of 5% for 72h. Thereafter, the supernatant was centrifuged at 2000 × g for 5 min to eliminate cellular fragments, aliquoted in cryotubes and stored at −80 °C.

The virus titer decay was evaluated using a plaque assay on Vero E6 cells performed for each experimental setting and the results compared with the positive control [38]. Briefly, each sample was serially diluted tenfold using DMEM (without FBS and Pen-Strep) and a 250 µL aliquot of each dilution was plated in triplicate in 24-wells plates containing 80%-90% confluent Vero cells. The viral inoculum was removed after 1.5 h of adsorption and 500 µL of DMEM (2% FBS; 0.75% carboxymethylcellulose; 1% Pen-Strep) were added. The 24-wells plates were incubated for 72h at 37 °C with a 5% CO_2 level. After 3 days, the medium was removed, cell monolayers were rinsed with 250 µL of phosphate buffered saline/well, fixed with 500 µL of formaldehyde at 4% vol./vol. and stained with 250 µL of crystal violet at 0.1% to highlight the presence of any plaque-forming units (pfu).

The potential cytotoxic effect of the peroxide solutions was evaluated by plating in quadruplicate 250 µL of each on Vero monolayer and incubating the 24-wells plates at 37 °C with CO_2 level of 5% for 1.5 h after pH neutralization and H_2O_2 disproportionation with catalase. The solutions were then removed and DMEM (2% FBS and 1% Pen-Strep) was added to the plate. After 3 days of incubation, cells monolayers were observed under an inverted microscope to detect any sign of

cytotoxicity. The same experimental protocol was used to investigate the possible cytopathic effect of catalase, citric acid, PC and HP, as single ingredients and in a mixture.

Residual inactivation activity was evaluated by neutralizing each solution with catalase, mixing with virus at a 1:1 ratio and inoculating on a 24-wells plate to determine virus titer, according to the European Standard NF EN 14476+A1. The viral concentration should be equivalent to the untreated original virus stock one (*i.e.*: a $\log_{10}$ difference ≤ 0.5).

A quantitative Real-Time – Polymerase Chain Reaction, qRT-PCR (quanty COVID-19, Clonit srl, Italy) was performed to estimate viral RNA concentration of untreated viral suspension and in samples with a significant after-treatment viral titer reduction. The assay detects three regions of SARS-CoV-2 nucleoprotein (N1, N2, N3), using four standards at a known titer of N1 (10^2, 10^3, 10^4, 10^5 copies μL^{-1}) to generate the quantification curve. Final RNA concentration was expressed as copies mL^{-1}. Briefly, all samples were extracted with the QIAsymphony DSP Virus/Pathogen Kit on the automatic QIAsymphony SP instrument (Qiagen, Hilden, Germany), following the manufacturer's instructions. The two amplification mix N1-N2 and N3-RP were prepared and added in a 96-well amplification plate along with 5 µL of each sample eluate; the qRT-PCR was then performed on the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems - ThermoFisher Scientific, USA).

All procedures involving the presence of SARS-CoV-2 were conducted in the biosafety level 4 laboratory (BSL4) of Clinical Laboratory of Microbiology, Virology and Bioemergency of "L. Sacco" University Hospital. This facility was chosen only to avoid interfering with routine diagnostic work, since a BSL3 environment would have been sufficient for tests on SARS-CoV-2 [S1].

^{13}C-NMR characterization

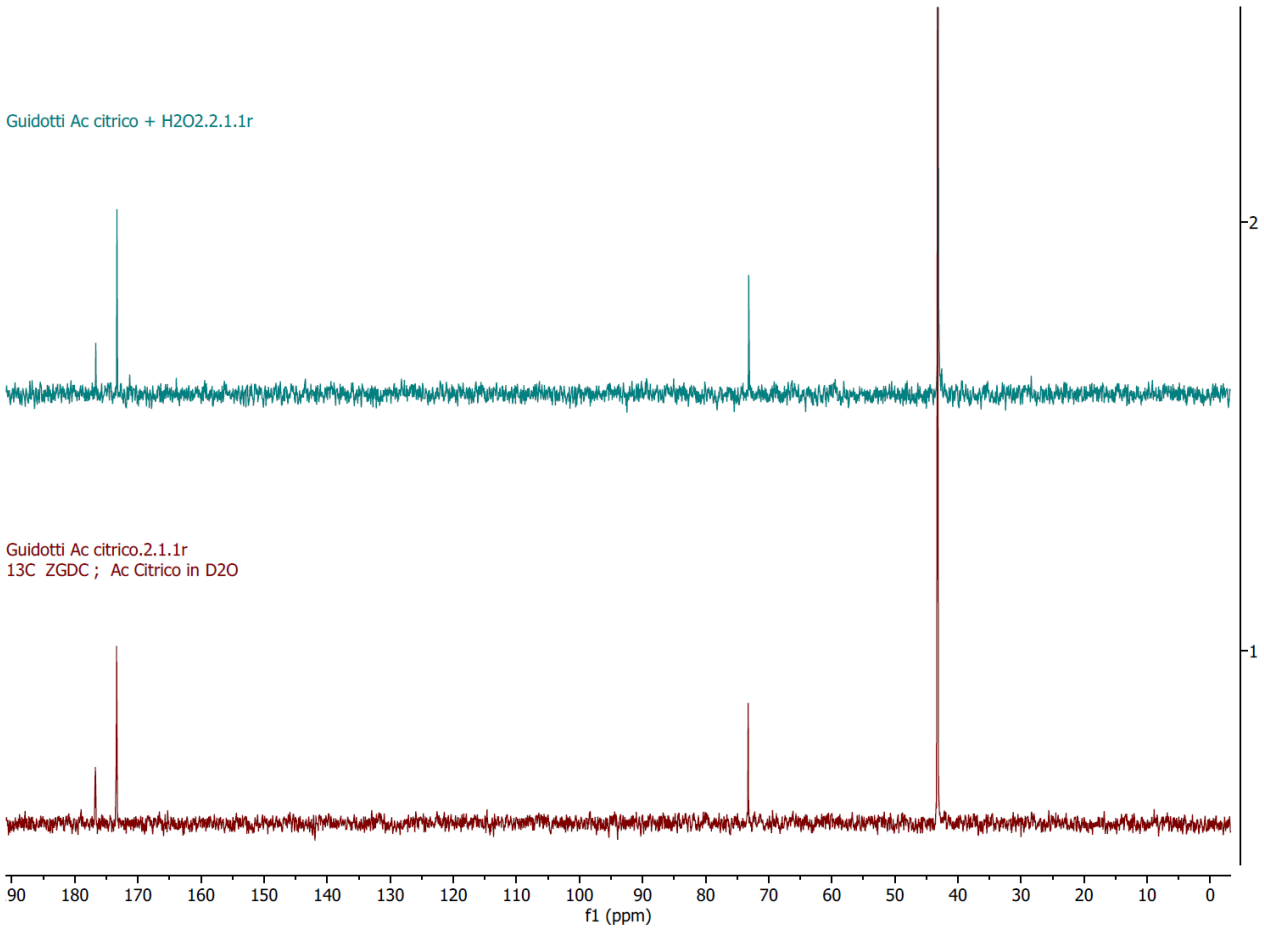

Figure S1. ^{13}C-NMR spectra of 1 mL solution of 20 mg of citric acid plus 79 µL of 35.1% aqueous hydrogen peroxide in D_2O (blue) and 1 mL solution of 20 mg of citric acid in D_2O (red). The mixture citric acid plus H_2O_2 was prepared at room temperature and left under stirring for 15 min.

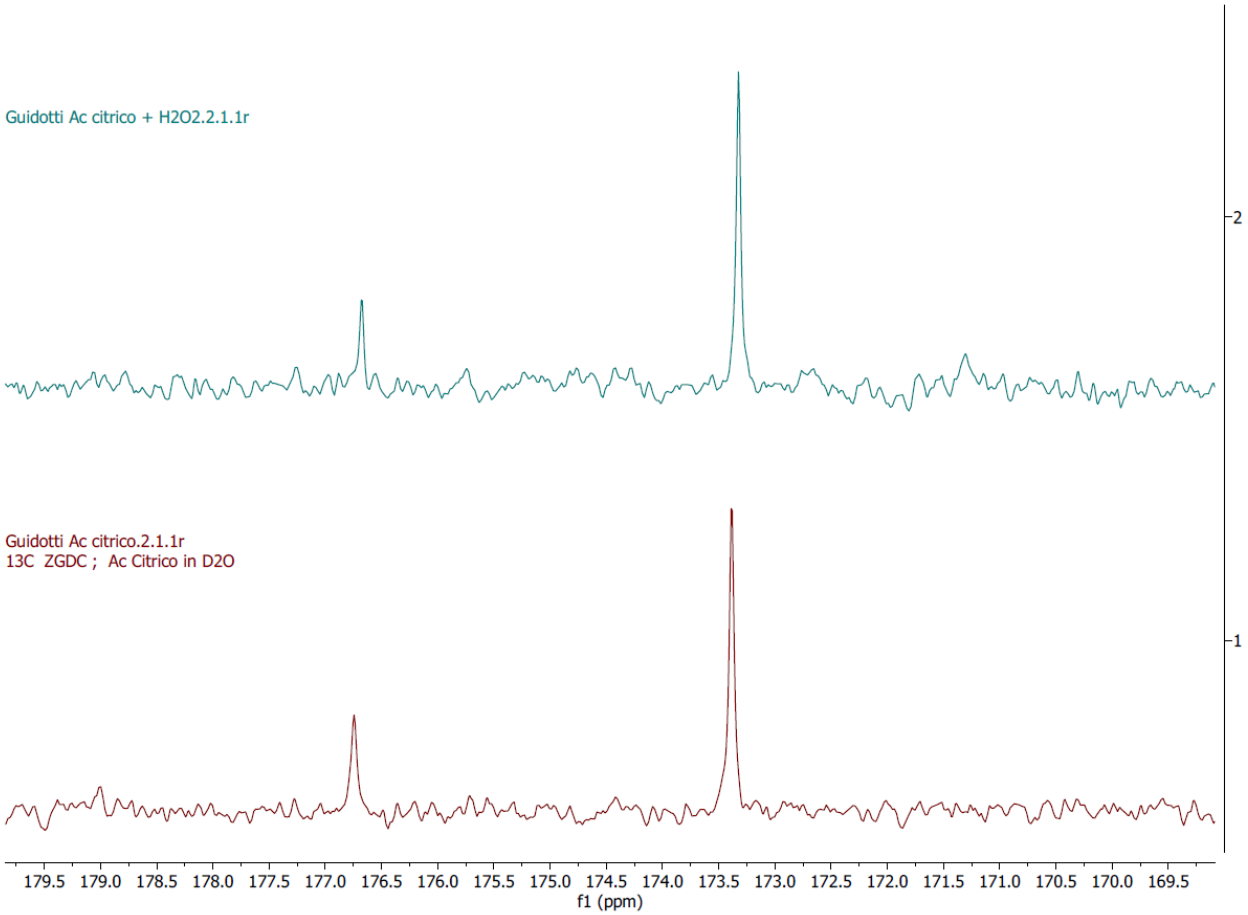

Figure S2. ^{13}C-NMR spectra of 1 mL solution of 20 mg of citric acid plus 79 µL of 35.1% aqueous hydrogen peroxide in D_2O (blue) and 1 mL solution of 20 mg of citric acid in D_2O (red). Magnification of the 169-180 ppm region, containing the signals of carboxyl carbon atoms.

In-vitro plaque assay

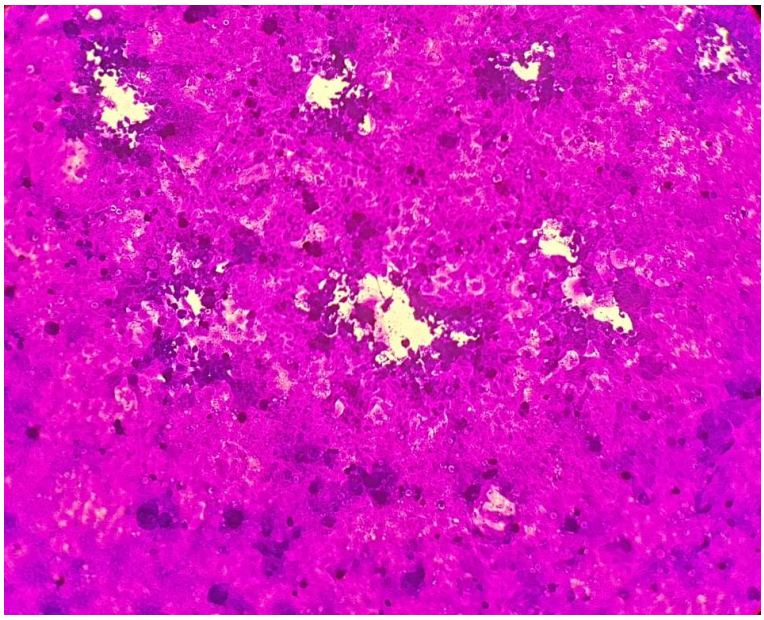

Figure S3. Formation of plaques on the cellular monolayer. Cell monolayers rinsed with 250 µL phosphate buffer, fixed with 500 µL 4% formaldehyde, stained with 250 µL 0.1% crystal violet and observed under inverted microscope, to highlight the presence of plaque-forming units.

Additional references

S1. World Health Organization; Laboratory biosafety guidance related to coronavirus disease (COVID-19): Interim guidance; 13 May 2020, WHO/WPE/GIH/2020.3